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GB 0915441 A EP 0253715 A2
Mechanisms of Ageing and Development 1983, 21, 121-136
J. Chromatography 1974, 88, 245-272

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(54) Collagens having methionine sulphoxide residues, processes for preparing them and cosmetic agents

(57) Collagens containing methionine sulphoxide residues, processes for preparing them and cosmetic agents are described, which cause no allergic skin reactions, and which may be used in cosmetic agents, particularly skin care products.

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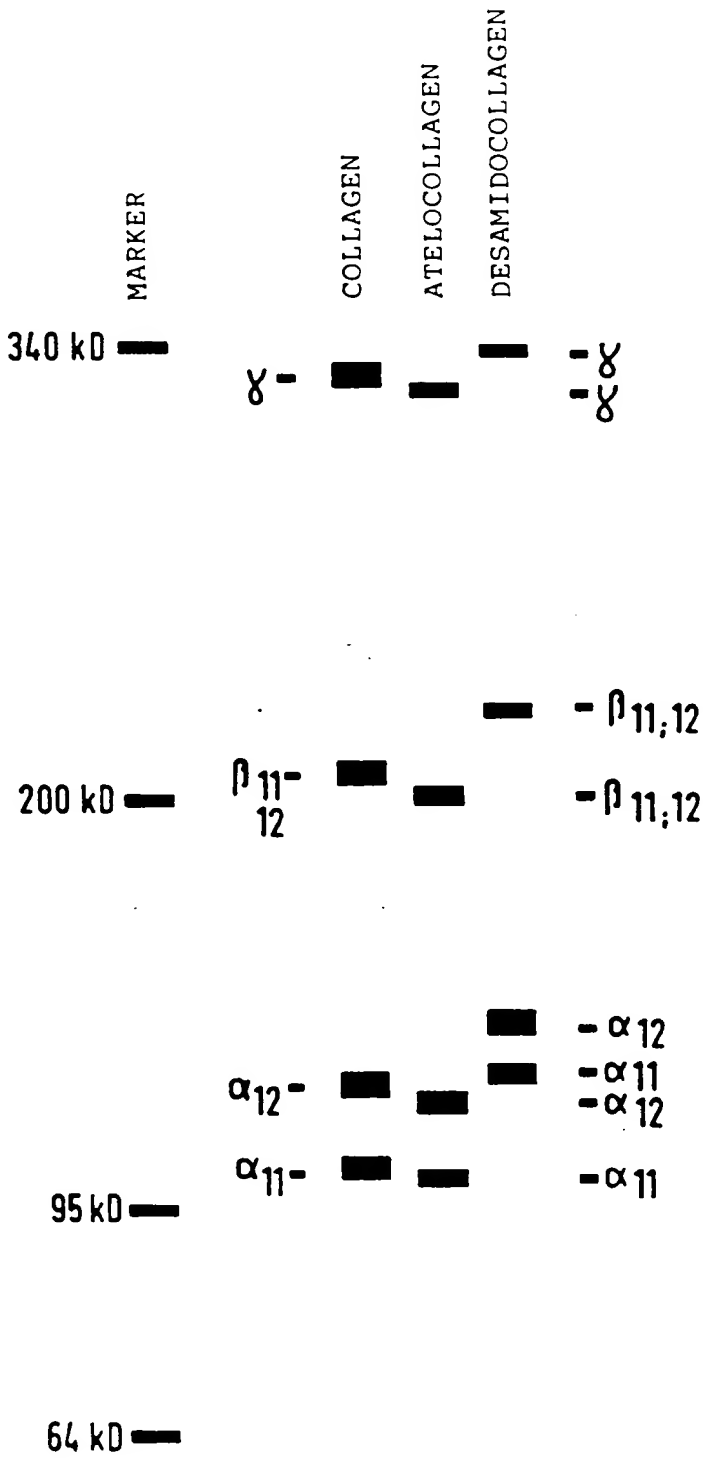
FIG. 1


MARKER
COLLAGEN
ATELOCOLLAGEN
DESAMIDOCOLLAGEN
340 kD
γ
200 kD
β 11-12
β 11;12
β 11;12
95 kD
α12
α12
α11
α11
α12
α11
64 kD

COLLAGENS, PROCESSES FOR PREPARING THEM AND COSMETIC AGENTS

The invention relates to collagens the methionine groups of which are at least partially sulphoxidised, processes for preparing them and cosmetic agents.

Collagen plays a very important part in the connective tissue of mammals. It makes up 25 to 30% of the total protein in the body. It is found particularly in skin, tendons, ligaments, cartilage, blood vessels, tendons and bones.

Tests have shown that collagen is not a uniform compound. Hitherto, at least twelve different types have been characterised. The various types of collagen which have different properties and also have different biological functions in the body, are characterised particularly by amino acid analysis, amino acid sequence, disulphide bridges between the chains, the length of the triple helix and their behaviour in an electrical field. By localisation with monoclonal antibodies it has been possible to investigate their distribution in various tissues. Thus, collagen type I and type III have been found particularly in the skin. These two types of collagen have a very similar amino acid composition.

Because of its amino acid composition, collagen assumes a special place among the proteins. It is particularly rich in glycine. In addition, it is the only protein to contain the amino acid hydroxyproline. The sulphur content is relatively low, since collagen type I, for example, contains no cysteine, and methionine is the only amino acid present which contains sulphur.

The collagen molecules are synthesised as procollagen in the fibroblasts and are released into the intercellular space. Here, the procollagen is converted

into collagen, with cleaving of the procollagen peptides. By crosslinking between different collagen molecules, insoluble collagen is finally produced, such as is found particularly in older connective tissue. Collagen generally consists of three polypeptide chains (molecular weight about 100,000 per chain), which when viewed individually form a left-handed helix and are twisted together to form a right-handed triple helix. The two ends of the molecule consist of non-helical telopeptides. These telopeptides play an important part in the natural crosslinking of the collagen molecules. They can easily be split off enzymatically (e.g. by pepsin or trypsin). The biochemistry, biology, biosynthesis and metabolism of collagen have been described in numerous scientific studies and surveys (see Appendix 1; reference is hereby made to the publications listed therein).

Collagen has a number of positive properties. Owing to its high water binding capacity it plays an important part in regulating the moisture in the skin. It also has a smoothing and anti-irritant effect. It stimulates the growth of the fibroblasts and accelerates wound healing. In addition, it also has haemostatic properties.

Owing to its positive properties and the fact that it is well tolerated, collagen is nowadays used in a number of indications in medicine (e.g. for suturing material, implant material, wound coverings, blood staunching).

For cosmetic purposes collagen can be extracted from various collagen sources. Principally, it is obtained from young or embryonic animal connective tissue. Preferably, calf skin is used, the collagen of which hardly differs from human collagen in physiological terms. Collagen obtained from calf skin consists of at least 90% of collagen type I and in addition it contains a small amount of collagen type

III. It is used either unchanged in the form of native soluble collagen, enzymatically changed in the form of a telocollagen or collagen hydrolysate or chemically changed in the form of desamidocollagen, collagen methylester, succinylated or guanidated collagen.

The methods of extraction and enzymatic and chemical modification are known and have been described in a number of publications and patents. An important point is to prevent the denaturing of the helical collagen structure. Furthermore, the work must be done under conditions which are as sterile as possible (see Appendix 2; reference is hereby made to the publications listed therein).

DE-AS 20 64 604 and the corresponding US Patent 3 991 184 describe skin care agents which contain native soluble collagen with an unchanged, substantially uncrosslinked collagen structure. The native soluble collagen is obtained from young or embryonic animal skin by extraction in slightly acidic aqueous media at low temperatures. The molecular weight of the collagen obtained in this way ranges from 5000 to 50,000.

The problem on which the invention is based is to prepare cosmetic active substances based on collagen which have a defined type of activity and exhibit no undesirable side effects. Furthermore, they should be simple and economical to produce.

Surprisingly, it has now been found that collagens with sulphoxidised methionine groups have advantageous cosmetic properties.

The invention therefore relates to collagens which are characterised in that at least some of the methionine groups contained in the collagen are present in the form of methionine sulphoxide. Otherwise, the collagen derivatives according to the invention are unchanged from the collagens used as starting material. The collagen derivatives according to the invention are virtually no longer sensitive to oxygen, because of the

sulphur atoms which have already been oxidised. Moreover, they are more water-soluble than non-derivatised collagens, particularly in acidic aqueous media, which means that the collagen derivatives according to the invention can be incorporated into the final products in higher concentrations.

The collagen derivatives according to the invention are exceptionally well tolerated by the skin; no allergic reactions have been encountered. They form a protective coating on skin and hair.

The collagen derivatives according to the invention may be prepared from any conventional collagen sources. It is preferable to use calf skin, the collagen of which is physiologically hardly distinguishable from human collagen, or native soluble collagen, collagen consisting of at least 90% of collagen type I, collagen type I or III, atelocollagen or desamidocollagen. It is also possible to use intermediate products from the collagen-processing industry.

The collagens according to the invention are produced by treating collagen-containing material (e.g. calf skin or intermediate products from the collagen-processing industry) in the presence of an oxidising agent to obtain collagen. If it is desired to produce a derivatised collagen, derivatisation is carried out at the same time. Accordingly, in the preparation of atelocollagen from calf skin, enzymatic degradation is carried out using proteases, e.g. trypsin or pepsin, in the presence of the oxidising agent, so as to obtain sulphoxidised atelocollagen. Similarly, in the preparation of desamidocollagen, the desamidation is carried out in the presence of the oxidising agent, so as to obtain sulphoxidised desamidocollagen.

The collagens according to the invention may also be prepared from isolated collagen or isolated derivatised collagen, by treating it with a suitable oxidising agent.

Suitable oxidising agents are those which bring about sulphoxidation of the sulphur atoms of the methionine groups contained in the collagen, without otherwise changing the collagen. Examples include hydrogen peroxide or alkali metal peroxides such as sodium peroxide or sodium perborate, peracids and peresters; hypohalites such as alkali metal hypohalites, particularly alkali metal hypochlorite, e.g. sodium hypochlorite and compounds which release hypohalites in situ; and chloramines, e.g. chloramine T. The oxidising agent is used in a quantity of 1.5 to 2.0 equivalents per equivalent of methionine. If necessary, excess oxidising agent is destroyed at the end of the reaction in the usual way by adding a reducing agent or removed by precipitating the collagen with NaCl and dissolving it again with citrate buffer.

The reaction is preferably carried out in an acid medium, more particularly at pH 2 to 5, most advantageously at pH 3 to 4. Expediently, a buffer, e.g. a citrate buffer, is added in order to keep the pH within the desired range.

The reaction temperature is preferably in the range from 0 to 20°C, more particularly 10 to 20°C and especially 15 to 17°C. The oxidising agent is generally left to act on the collagen for at least one day, generally 2 to 6 days, preferably 3 to 4 days.

Prepferably, the collagen-containing material is pretreated with the oxidising agent, e.g. by spraying or dipping in a solution of the oxidising agent. 0.05 to 0.5 equivalents of oxidising agents are preferably used per kg of collagen-containing material (about 30% collagen content) in 0.05 to 0.5% by weight aqueous solution. Pretreatment is preferably carried out for 1 to 10 hours, more particularly 2 to 5 hours. The work is preferably done at a temperature in the range from 20 to 50°C, more particularly 30 to 50°C, or the collagen-containing material is sprayed, in the deep-frozen

state, with the oxidising agent and left to thaw.

Under the mild conditions described above, sulphoxidation of the methionine groups in the collagen takes place, without the corresponding sulphone being formed. The yield is 91 to 96%, based on the collagen used. The sulphoxidation is reversible; for example, the derivatives according to the invention can be converted back into the starting materials by reacting with mercaptoethanol.

Collagens generally contain 5 to 6 methionine groups per 1000 amino acids. Of these, 2 to 5, preferably 2 to 3 methionine groups are sulphoxidised in the collagens according to the invention (see the Table hereinafter).

The collagens according to the invention have a molecular weight of about 270,000 to about 300,000 (determined by SDS-polyacrylamide gel electrophoresis under the conditions specified by U.K. Laemmli, Nature 227, 680-685 (1970)).

The collagens can be further characterised and their purity determined by the usual biochemical and physico-chemical methods, e.g. HPLC, SDS-polyacrylamide gel electrophoresis, amino acid analysis, nitrogen analysis, peptide mapping techniques, cyanobromide cleaving, optical rotation dispersion. One particular possibility for determining the content is to measure the content of the characteristic amino acid hydroxypyroline. Its proportion in collagen from the skin is 14% (Nimni, M. E., Nato Asi, Ser. E. 116, 365-383 (1986)).

In the process according to the invention the products are obtained in aqueous solution. This solution may be processed as it is, optionally after concentration, to produce cosmetic agents. The solutions are obtained in virtually sterile form owing to the use of the oxidising agent, so that the use of preservatives can be omitted if desired.

The collagen derivatives according to the invention can be obtained in the usual way from the above-mentioned solutions, e.g. by spray drying, lyophilising, etc. The solid products obtained in this way can optionally be processed to form cosmetic agents.

In a particularly preferred process, hide from slaughtered calves is used as starting material. The hides are preferably used deep-frozen and disinfected, e.g. with chloramine T.

The hides are then cut into pieces either after thawing or, preferably, in the deep-frozen state. During the cutting up or subsequently, the hides are pretreated with the oxidising agent used according to the invention under the conditions specified hereinbefore.

The pretreated pieces of hide are then comminuted into a slurry in a mincing machine. The slurry is then treated with the oxidising agent under the conditions specified above. Preferably, the slurry is extracted with citrate buffer with stirring at 15 to 17°C. It is particularly preferred that the concentration of oxidising agent, especially hydrogen peroxide, be kept constant throughout the extraction step, e.g. at a level of 80 to 120 ppm, more particularly 90 to 110 ppm.

The slurry is then filtered through filter bags of doubly pre-shrunk cotton filter material. If desired, excess oxidising agent is destroyed in the usual way. The product thus obtained has a molecular weight of about 295,000 and the gel electrophoresis chromatogram shown in Figure 1. The amino acid analysis of this product is shown in the Table which appears hereinafter.

The invention also relates to cosmetic agents containing the collagen derivatives according to the invention. These cosmetic agents are, chiefly, skin care products which may take the form of creams, masks, packs, lotions, gels, milks, etc. For this purpose the collagen derivatives according to the invention are

incorporated in conventional cosmetic bases and excipients.

Examples of suitable bases include oils such as Vaseline oil, avocado oil, paraffin oil and the like. Excipients which may be used include, for example, emulsifiers such as mixtures of oils and/or fatty alcohols or polyethoxylated alcohols, soaps and the like; thickeners such as sodium alginate, gum arabic, xanthine gum, cellulose derivatives and the like; propellants for formulating aerosols, such as carbon dioxide and nitrogen; solvents such as alcohols and the like.

The agents according to the invention may also contain the ingredients conventionally used in cosmetics. These include for example perfumes, dyestuffs, preservatives, antioxidants, sequestering agents, plasticisers, emulsifiers and the like.

The agents according to the invention may also contain cosmetically active additives. These include for example moisture retaining agents, carotinoids, stabilisers, moisture regulators, pH regulators, UV-A and UV-B filters and the like.

The agents according to the invention generally contain about 0.01 to 5%, preferably about 0.1 to 2% by weight of collagen derivatives, based on the total weight of the agent.

The Examples which follow are intended to illustrate the preparation of the active substances according to the invention.

Example 1

a) 250 kg of deep-frozen calf skins (masks; disinfected with chloramine T) are coarsely chopped up in the deep-frozen state and at the same time sprayed with a solution of 2500 ml of 30% hydrogen peroxide in 500 l of water. The pieces are then left to thaw (about 2

to 3 hours). The water which has collected is drained off and the pieces are optionally washed once more with 500 litres of water. After the water has been drained off the pieces of skin are passed through a mincer.

100 litres of 0.128 m citrate buffer (pH 3.6 at 15 to 17°C) and 400 ml of 30% hydrogen peroxide are placed in a 400 litre vessel. Then 50 kg of minced (comminuted) tissue are added. After 30 minutes stirring at 15 to 17°C the concentration of hydrogen peroxide is measured. By further addition, the concentration of hydrogen peroxide is maintained at about 100 ppm. Using this process, solutions are obtained which contain 0.4 to 0.8% dissolved collagen (corresponding to 600 g to 1200 g per batch). The constancy of the hydrogen peroxide concentration (in this case 100 ppm, corresponding to 40 ml per 150 litre batch) is of crucial importance, so that collagen going into solution which has not yet reacted in the heterogeneous phase is able to react to produce collagen with sulphoxidised methionine. Moreover, this concentration of hydrogen peroxide produces an ideal bacteriostatic medium. The suspension is stirred for 3 days at 15 to 17°C, then diluted with 100 litres of buffer and stirred for 24 hours.

By separation at 10,000 rpm, a solution of native soluble collagen is obtained which is slightly opalescent and this is pooled with other extracts so that the solution contains 0.225% soluble collagen (300 µg hydroxyproline/ml). Excess hydrogen peroxide is decomposed by known methods.

As an alternative, in order to eliminate hydrogen peroxide, the centrifuged material is mixed with common salt until the saline concentration is 6% by weight, the fibrils precipitated are separated by centrifuging and finally dissolved in 0.128 molar citrate buffer.

Conventional preservatives are used, e.g. Phenonip (mixture of p-hydroxybenzoic acid esters with phenoxyethanol). The molecular weight of the sulphoxidised collagen is about 295,000 (determined by SDS-polyacrylamide gel electrophoresis). The product is characterised in that methionine sulphoxide emerges before aspartic acid in the elution diagram in amino acid analysis and later, correspondingly less methionine can be detected.

For amino acid analysis, one aliquot of the solution is removed and mixed with common salt until the solution contains 6% by weight of common salt. The collagen precipitated is removed by centrifuging, dialysed three times with 0.1 m acetic acid and freeze-dried.

For the amino acid analysis, 5 mg of freeze-dried collagen are hydrolysed in 10 ml of 6 N HCl under nitrogen for 2 hours at 110° ± 1°. The excess hydrochloric acid is removed in vacuo and the residue is taken up in elution buffer, and the amino acids are measured by ion exchange chromatography.

Table 1 Amino acid composition (groups per 1000 amino acids)

	Collagen Type I	Collagen Type III	Example 1 (sulphoxidised native collagen)	Example 2 (sulphoxidised atelocollagen)	Example 3 (sulphoxidised desamidocollagen)
Aspartic acid	45	48	47	48	49
Methionine sulphoxide	-		3	3	2
Methionine sulphone	-		-	-	-
Hydroxyproline	93	118	84	86	84
Threonine	17	15	17	17	19
Serine	33	37	33	35	36
Glutamic acid	74	70	77	77	80
Proline	132	105	130	127	125
Glycine	323	344	303	332	307
Alanine	107	90	114	111	113
Valine	21	14	23	23	24
Methionine	5	5	3	3	4
Isoleucine	13	12	19	13	14
Leucine	24	18	40	26	30
Tyrosine	2	2	1.5	5	6
Phenylalanine	12	8	8	8	9
Lysine	29	28	30	32	30
Histidine	5	8	8	8	6
Arginine	52	46	52	54	47

b) Native soluble collagen can be converted into a corresponding collagen with sulphoxidised methionine under the reaction conditions specified above (1000 g of native soluble collagen in 100 litres of 0.128 M citrate buffer, pH 3.6, and 400 ml of hydrogen peroxide; temperature and working conditions as specified in Example 1).

Example 2

a) The skin residues from Example 1 or freshly comminuted skin can be reacted, as described in Example 1, in the presence of hydrogen peroxide in an enzymatic reaction with trypsin or pepsin (for literature see Appendix 2) to obtain atelocollagen with sulphoxidised methionine. For the amino acid composition see Table 1; molecular weight of the product: ~ 270,000 (SDS-polyacrylamide gel electrophoresis)

b) Pure atelocollagen can be converted into atelocollagen with sulphoxidised methionine in a similar way and under the reaction conditions specified in Example 1b)

Example 3

a) The skin residues from Example 1 or freshly comminuted skin can be reacted as described in Example 1 in the presence of hydrogen peroxide with alkali or acids (for literature see Appendix 2) to obtain desamidocollagen with sulphoxidised methionine. For amino acid composition see Table 1; molecular weight of the product: ~ 295,000 (SDS-polyacrylamide gel electrophoresis).

b) Pure desamidocollagen can be converted into

desamidocollagen with sulphoxidised methionine in a similar way and under the reaction conditions specified in Example 1b).

Figure 1 shows the SDS-polyacrylamide gel electrophoresis chromatograms for the products obtained in Examples 1 to 3. The gel electrophoresis was carried out on 7% gel under the conditions specified by U.K. Laemmle in Nature, 227, 680 to 685 (1970). Under these conditions, there is partial cleaving of the triple helix of the collagens, so that the chromatogram shows the γ-chains with a molecular weight of about 300 KD, the β-chains with a molecular weight of about 200 KD and the α-chains with a molecular weight of about 100 KD. No proteolytic breakdown products can be detected.

In the Examples which follow, the collagen derivatives of Examples 1 to 3 can be used in the form of the solution obtained or in solid form.

Example 4

Skin cream:

Collagen derivative	0.1 g
Polyoxyethylene cetylether	1.5 g
Cetyl alcohol	2 g
Vaseline oil	6 g
Avocado oil	4 g
Lanolin	4 g
Perfume, as necessary	
Sterile water ad	100 g

Example 5

Body milk:

Collagen derivative	1 g
Paraffin oil	5 g
Vaseline oil	7 g

Preservative	0.15	g
Triethanolamine stearate	5	g
Stearic acid	3	g
Sterile water ad	100	g

Example 6

Skin lotion:

Collagen derivative	0.5	g
Preservative	0.15	g
Polyvinylpyrrolidone	3	g
Ethanol	20	g
Sterile water ad	100	g

Example 7

Skin gel:

Collagen derivative	0.5	g
Preservative	0.15	g
Ethanol	40	g
Propyleneglycol	42	g
Acrylic acid polymer (Carbopol 940 made by Goodrich Chemical Co.)	1	g
Sterile water ad	100	g

Appendix 1

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4 687 518

Claims:

1. Collagens, characterised in that at least some of the methionine groups contained in the collagen are present in the form of methionine sulphoxide.

2. Collagens according to claim 1, characterised in that they are based on collagen which is selected from native soluble collagen, atelocollagen, desamidocollagen or a collagen consisting of at least 90% of collagen type I.

3. Collagens according to claim 1 or 2, characterised in that 2 to 5 methionine groups per 1000 collagen amino acids are in the form of methionine sulphoxide.

4. Collagens according to claim 3 characterised in that 2 to 3 methionine groups per 1000 collagen amino acids are in the form of methionine sulphoxide.

5. Process for preparing the collagens according to claim 1, characterised in that collagen-containing material is treated with a suitable oxidising agent to produce collagen or derivatised collagen or isolated collagen or isolated derivatised collagen.

6. Process according to claim 5, characterised in that hydrogen peroxide or an alkali metal peroxide is used as the oxidising agent.

7. Process according to claim 5 or 6, characterised in that the oxidising agent is used in a quantity of 1.5 to 2.0 equivalents per methionine equivalent.

8. Process according to claim 7 characterised in that the oxidising agent is used in a quantity of 1.6 to 1.9 equivalents per methionine equivalent.

9. Process according to one of claims 5 to 8, characterised in that calf skin, native soluble collagen, atelocollagen or desamidocollagen is used as the starting material.

10. Process according to one of claims 5 to 9, characterised in that the reaction is carried out in an acid medium.

11. Process according to claim 10 characterised in that the reaction is carried out at a pH in the range from 2 to 5.

12. Process according to claim 11 characterised in that the reaction is carried out at a pH in the range from 3 to 4.

13. Process according to one of claims 5 to 12, characterised in that reaction is carried out at a temperature in the range from 0 to 20°C.

14. Process according to claim 13 characterised in that the reaction is carried out at a temperature in the range from 10 to 20°C.

15. Process according to one of claims 5 to 14, characterised in that the collagen-containing material is pretreated with the oxidising agent.

16. Process according to claim 15, characterised in that the collagen-containing material is comminuted in the deep-frozen state before pretreatment.

17. Cosmetic agent, containing at least one collagen according to any of claims 1 to 3 or a collagen obtained according to any of claims 5 to 16.

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